(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
27 February 2003 (27.02.2003)

PCT

(10) International Publication Number
WO 03/016538 A1

(51) International Patent Classification⁷: C12N 15/70

(21) International Application Number: PCT/KR02/01547

(22) International Filing Date: 13 August 2002 (13.08.2002)

(25) Filing Language: Korean

(26) Publication Language: English

(30) Priority Data:
2001/48881 14 August 2001 (14.08.2001) KR

(71) Applicant *(for all designated States except US)*: **KOREA ADVANCED INSTITUTE OF SCIENCE AND TECHNOLOGY** [KR/KR]; 373-1 Kusong-dong, Yusong-gu, 305-701 Taejon (KR).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: **LEE, Sang-Yup** [KR/KR]; 212-702 Expo Apartment, 464-1 Jeonmin-dong, Yusong-gu, 305-390 Taejon (KR). **JEONG, Ki-Jun** [KR/KR]; 102-411 Kaist Apartment, 392-3 Kung-dong, Yusong-gu, 305-335 Taejon (KR).

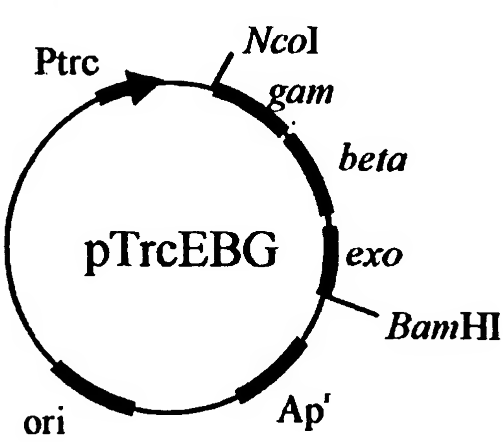
(74) Agent: **LEE, Han-Young**; 8th Fl., Seowon Bldg. 1675-1 Seocho-dong, Seocho-gu, 137-070 Seoul (KR).

(81) Designated States *(national)*: CN, JP, US.

Published:
— *with international search report*
— *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: A METHOD OR EXTRACELLULAR PRODUCTION OF TARGET PROTEINS EMPLOYING OMPF IN E. COLI



(57) Abstract: The present invention provides an expression vector comprising a gene coding for OmpF protein in E. coli, E.coli transformed with the said expression vector, and a method for extracellular production of target proteins employing the said microorganism. The recombinant expression vector of the invention comprises ampicillin-resistant gene, OmpF promoter and OmpF gene. In accordance with the invention, a target protein can be produced extracellularly by simpler method than conventional methods in a manner that: secretory production of OmpF fusion protein begins simultaneously with growth of cells due to constitutive expression employing OmpF promoter, and as the concentration of cells increases, the amount of secretory production of the protein also increases continuously. Therefore, target proteins can be produced in large quantities by a high concentration culture of cells.

WO 03/016538 A1

A METHOD FOR EXTRACELLULAR PRODUCTION OF TARGET PROTEINS EMPLOYING OmpF IN *E.coli*

BACKGROUND OF THE INVENTION

Field of the Invention

The present invention relates to a method for extracellular production of desired proteins employing outer membrane protein F(OmpF) of *Escherichia coli*(*E. coli*), more specifically, to an expression vector comprising genes encoding OmpF and desired protein, *E. coli* transformed with the expression vector, and a method for extracellular production of desired proteins by employing the same.

Description of the Prior Art

It has been well known that extracellular production of desired foreign proteins in *E. coli* is a very efficient method in the senses that: the secreted foreign proteins are protected against proteolysis by proteolytic enzymes in *E. coli*, the secretion process guides appropriate folding of foreign proteins to inhibit the formation of insoluble inclusion bodies, and N-terminal secretion signal peptide is removed from foreign proteins during secretion process to keep the amino acid sequence identical to the naturally occurring one. This method also allows mass production of foreign proteins through high concentration culture and continuous culture. Furthermore, this method makes for pure purification of foreign proteins because little bacterial proteins are secreted into culture media.

Since the extracellular production has several advantages as aboves, various studies on the extracellular production systems have been actively made to produce

desired foreign proteins in *E. coli*. The extracellular production systems developed so far are classified as the following three categories: the first one is a method for extracellular production by the recombination of secretion signal sequence and desired foreign protein. For example, Toksoy et al. produced *Taq*I protein on the cell surface employing a fusion protein of a secretion signal sequence and maltose binding protein(MBP), Lo et al. produced β-1,4-endoglucanase of *Bacillus subtilis* on the cell surface of *E. coli*, and Nagahari et al. produced β-endorphine on the cell surface of *E. coli* through the recombination of OmpF secretion signal peptide and 8 amino acids from N-terminal of OmpF. In addition, Yamamoto et al. tried to produce p21 protein of harvey murine sarcoma virus extracellularly by using OmpF secretion signal sequence. However, it turned out that p21 was not produced on the cell surface, but accumulated in inclusion bodies(see: Toksoy E. et al., Biotechnology Techniques, 13:803-808, 1999; Lo A. C. et al., Appl. Environ. Micrbiol., 54:2287-2292, 1988; Nagahari et al., EMBO J., 4:3589-3592, 1985; and, Yamamoto et al., Appl. Micobiol. Biotechnol., 35:615-621, 1991). The second one is a method for extracellular production by the recombination of secretion protein of *E. coli* and desird protein. For example, Baneyx et al. produced OmpA-TEM-β-lactamase fusion protein on the cell surface together with TolAIII membrane protein of *E. coli*, Robbens et al. used *kil* gene to produce interleukin-2, van der Wal et al. used a lipoprotein, BRP(bacteriocin release protein), to produce β-lactamase on the cell surface, and Aristidou et al. increased the yield of extracellular production using BRP by addition of glycine to culture media(see: Baneyx F. and Eugene W. M., Protein Expr. Purif., 14:13-22, 1998; Robbens J. et al., Protein Expr. Purif., 6:481-486, 1995; van der Wal F. J. et al., Appl. Environ. Microbiol., 64:392-398, 1998; and, Aristidou A. A. et al., Biotechnol. Lett., 15:331-336, 1993). The third one is a method for extracellular production by the aid of

outer membrane-free *E. coli*, i.e., L-type strain of *E. coli*, a mutant that has only inner cellular membrane without outer cellular membrane and periplasm. The extracellular production of foreign proteins is simpler than prior methods in the culture of L-type strain because expressed proteins are transported through only inner cellular membrane to be secreted into culture media. For example, Kujau et al. used RV308 strain, a L-type mutant, to produce miniantibody(miniAb) on the cell surface(see: Kajau M. J. et al., Appl. Microbiol. Biotechnol., 49:51-58, 1998).

As expounded as aboves, a variety of methods have been developed to produce desired foreign proteins on the cell surface of *E. coli*. Most of these prior art methods are, however, proven to be less satisfactory in a sense that partial degradation of some foreign proteins by bacterial proteolytic enzymes makes the purification process complex and high concentration cell culture impossible. In addition, extracellular production employing L-type strain of *E. coli* has a shortcoming that the said strain is not suitable for high concentration cell culture due to its weak resistance to environmental stress and its short life cycle.

Under the circumstances, there are strong reasons for exploring and developing an alternative method for extracellular production of desired foreign proteins on the cell surface of *E. coli*.

Summary of the Invention

The present inventors have made an effort to develop a novel method for extracellular production of desired foreign proteins on the cell surface of *E. coli* and found that: fusion proteins can be secreted efficiently into culture media of recombinant *E. coli* transformed with an expression vector comprising genes encoding outer membrane protein F(OmpF) of *E. coli* and desired protein, and the

foreign proteins can be purified in a simple manner by removing OmpF from the fusion proteins.

A primary object of the present invention is, therefore, to provide an expression vector comprising genes encoding OmpF of *E. coli* and desired foreign protein.

The other object of the invention is to provide a microorganism that is transformed with the expression vector.

Another object of the invention is to provide a method for extracellular production of desired protein by culturing the transformed microorganism.

Brief Description of Drawings

The above objects and features of the present invention will become apparent from the following descriptions given in conjunction with the accompanying drawings, in which:

Figure 1 represents a genetic map of recombinant expression vector pTrcEBG.

Figure 2 represents a genetic map of expression vector pOmpF6.

Figure 3 represents a genetic map of recombinant expression vector pSKOmpFKm.

Figure 4 represents a genetic map of recombinant expression vector pEDOmpF3.

Figure 5 represents a genetic map of recombinant expression vector pTrcOmpF4.

Figure 6 represents a construction scheme and a genetic map of recombinant

expression vector pOmpF6βE.

Detailed Description of the Invention

An expression vector of the present invention contains ampicillin resistant gene, OmpF promoter and OmpF gene.

The method for extracellular production of desired protein employing an expression vector comprises the steps of: introducing a gene encoding oligopeptide which is recognized and cleaved by a proteolytic enzyme and a gene encoding desired protein into the expression vector pOmpF6 to construct a recombinant expression vector producing the desired protein extracellularly; transforming a host microorganism lacking OmpF gene with the recombinant expression vector to obtain a transformed microorganism; culturing the transformed microorganism to produce OmpF-fused protein from the culture; and, treating the fused protein with a proteolytic enzyme and obtaining the desired protein. Available proteolytic enzyme includes Factor Xa, enterokinase(Asp-Asp-Asp-Asp-Lys), genenase(His-Tyr or Tyr-His), IgA protease(Pro/Ser-Arg/Thr-Pro-Pro-Thr /Ser/Ala-Pro), intein, thrombin, trypsin, pepsin and subtilisin or plasmin, preferably Factor Xa. Available desired protein includes peptide, enzyme and antibody that can be fused to OmpF, preferably β-endorphin. Microorganisms of *Escherichia* sp. or *Samonella* sp. is, but not limited these to, preferable host microorganism.

The present invention is further described in the following.

The present inventors cultured six *E. coli* strains (BL21(DE3), HB101, JM101, MC4100, XL1-Blue and W3110), analysed the outer membrane proteins separated from each culture by SDS-PAGE, and found that OmpF protein was over

expressed in BL21(DE3) strain. The present inventors constructed an expression vector pOmpF6, which consists of OmpF gene of *E. coli*, OmpF promoter, and ampicillin resistant gene. OmpF gene and OmpF promoter were cloned by perfoming PCR using genomic DNA isolated from BL21(DE3) as a template and specific primers. *E. coli* BL101 strain was transformed with the said expression vector and the transformant was designated as *E. coli* BL101/pOmpF6(*Escherichia coli* BL101/pOmpF6) and deposited with the Korean Collection for Type Cultures(KCTC, #52 Oun-dong, Yusong-ku, Taejon 305-333, Republic of Korea), an international depository authority, as Accession No. KCTC 1026BP on June 1, 2001.

Then, the present inventors constructed a recombinant expression vector pOmpF6βE to demonstrate an example of extracellular production of fusion proteins using the expression vector pOmpF6. The recombinant expression vector contains a cDNA encoding β-endorphin, a gene encoding OmpF protein, OmpF promoter, a gene encoding oligopeptide for the recognition and cleavage by Factor Xa, which is inserted between OmpF protein and β-endorphin, and ampicillin resistant gene. The present inventors transformed *E. coli* BL21(DE3) strain(Novagen Co., U.S.A.) with the said recombinant expression vector pOmpF6βE, cultured the said recombinant *E. coli* strain to produce OmpF-β-endorphin fusion proteins on the cell surface, and harvested fusion proteins from culture media. The said harvested fusion proteins were first purified by the anion-exchange chromatography and β-endorphin proteins were recovered after removing OmpF proteins from fusion proteins by Factor Xa.

High concentration cell culture is practically impossible in the conventional cell surface display systems since fusion proteins are degraded by proteolytic enzymes of *E. coli*. In accordance with the invention, a desired

protein can be produced extracellularly by simpler method than conventional methods in a manner that: secretory production of OmpF fusion protein begins simultaneously with growth of cells through constitutive expression employing OmpF promoter, and as the concentration of cells increases, the amount of secretory production of the protein also increases continuously. Therefore, desired proteins can be produced in large quantities by a high concentration culture of cells.

The present invention is further illustrated in the following examples, which should not be taken to limit the scope of the invention.

Example 1: Selection of *E. coli* strain over expressing OmpF

Six *E. coli* strains conventionally used for the production of recombinant proteins were selected, and outer membrane proteins were purified therefrom and compared with one another by carrying out SDS-PAGE. Six *E. coli* strains thus selected were *E. coli* BL21(DE3)[*F- ompT hsdSB(rB- mB-) gal dcm(DE3)* a prophage carrying the T7 RNA polymerase gene](Novagen Co., U.S.A.), HB101[*F- hsd20(rk-, mk-) recA13 ara-14 proA2 lacY1 galK2 rpsL20(str) xyl1-5 mtl-1 supE44 λ-*](New England Biolabs, U.S.A.), JM101[supE thi-1 Δ(lac-proAB) [F'traD36 proAB lacIqZ ΔM15]](Stratagene Co., U.S.A.), MC4100[*F- araD139 Δ(argF-lac)U169 rpsL150(strr) relA1 flbB5301 deoC1 ptsF25 rbsR*](Stratagene Co., U.S.A.), XL1-Blue[*SupE44 hsdR17 recA1 endA1 gyrA96 thi relA1 lacF(proAB+ lacIq lacZM15 Tn10(tetr)*](Stratagene Co., U.S.A.), and W3110[derived from K-12, λ-, F-, prototrophic](KCTC 2223). Each of *E. coli* strains was cultured in 50mL of LB media(tryptone 10g/L, yeast extract 5g/L, NaCl 5g/L) at 37°C.

Bacterial cells were harvested from each of the cultures and the outer membrane proteins were fractionated

by the following process: Bacterial cells were first harvested by centrifuging 3mL of the culture at 3500 x g for 5 min at 4°C. The harvested bacterial cells were washed with 1mL of Na_2HPO_4(pH 7.2) buffer solution, centrifuged again at 3500 x g for 5 min at 4°C, and suspended in 0.5mL of Na_2HPO_4(pH 7.2) buffer solution. The bacterial cell suspension was treated by sonication and centrifuged at 10,000 x g for 2 min at room temperature to remove the cell debris. The supernatant was centrifuged at 10,000 x g for 30 min at room temperature and the pellet was suspended in 0.5mL of 10M Na_2HPO_4(pH 7.2) buffer solution containing 0.5%(w/v) sarcosyl to prepare the fraction of the outer membrane proteins.

The fraction was incubated at 37°C for 30 min and centrifuged at 10,000 x g for 30 min at 4°C. The pellet was washed with 10mM Na_2HPO_4(pH 7.2) buffer solution and suspended in 50μl of PBS(0.247M NaCl, 0.041M Na_2HPO_4, 0.047M KH_2PO_4, 0.005M KCl, pH 7.4) to prepare the fraction sample of the outer membrane proteins for the protein analysis(see: Puenete, J.L. et al., Gene, 156:1-9, 1995). Each of fraction samples was analysed by SDS-PAGE, showing that *E. coli* BL21(DE3) strain produced a large amount of OmpF protein.

Example 2: Preparation of *E. coli* strain lacking ompF gene

ompF gene of *E. coli* BL21(DE3) was deleted by using red operon(exo-beta-gam) of bacteriophage: First, PCR was performed by empolying bacteriophage DNA as a template and primer pair of primer 1: 5'-CGCGCCATGGATATTAATACTGAAACTGAGATCAAGC-3'(SEQ ID NO. 1) and primer 2: 5'- CGGGATCCTCATCGCCATTGCTCCCCAAATAC-3'(SEQ ID NO. 2). The amplified PCR product was separated on 1.2% agarose gel by electrophoresis to obtain 2.2 kb DNA fragment. The DNA fragment was digested with *Nco*I and *Bam*HI restriction enzymes. The expression plasmid pTrc99A(Pharmacia Biotech Co., U.S.A.) containing *trc*

promoter was also digested with *Nco*I and *Bam*HI restriction enzymes, and ligated to the PCR product digested with the same restriction enzymes to construct a recombinant expression vector pTrcEBG. Then, *E. coli* XL1-Blue strain was transformed with the expression vector pTrcEBG and transformants were screened on LB plate containing 50μg/L ampicillin(see: Fig. 1). Figure 1 represents a genetic map of recombinant expression vector pTrcEBG. *E. coli* BL21(DE3) strain was transformed with the recombinant expression vector pTrcEBG and transformants were screened on LB plate containg 50μg/L ampicillin. The transformant was cultured in 500mL of LB media until $O.D._{600}$ reached to 0.3 and IPTG(final concentration 1mM) was added into media to induce the expression of exo-beta-gam gene. After culture for 1 hr, bacterial cells were harvested by centrifugation and washed with 250mL of deionized distilled water. The harvested cells were suspended in 10mL of 10%(w/v) glycerol and stored at -80°C after centrifugation.

On the other hand, PCR was performed using genomic DNA of *E. coli* BL21(DE3) strain as a template and primer pair of primer 3: 5'-CGGAATTCTGGATTATACCGACGCAG-3'(SEQ ID NO. 3) and primer 4: 5'-GCGGATCCTTAGAACTGGTAAACGATAC-3'(SEQ ID NO. 4) to obtain 2,160bp DNA fragment. The DNA fragment was digested with *Eco*RI and *Bam*HI restriction enzymes and inserted into pBluescript SK(-)(Stratagene Cloning Systems, U.S.A.) to construct an expression vector pOmpF6. Then, *E. coli* XL1-Blue strain was transformed with the expression vector, and then transformants were screened on LB plate containing 50μg/L ampicillin(see: Fig. 2). Figure 2 represents a genetic map of expression vector pOmpF6. In addition, PCR was performed using an expression vector pACY177(New England Biolabs, U.S.A.) as a template and primer 5: 5'-CGCTGCAGTTAGAAAAACTCATCGAGCATC-3'(SEQ ID NO. 5) and primer 6: 5'-GCCTGCAGGCCACGTTGTGTCCTCAAA-3'(SEQ ID NO. 6) to obtain 940bp DNA fragment. The DNA fragment was digested

with *Pst*I restriction enzyme and ligated into *Pst*I-digested plasmid pOmpF6 to construct a recombinant expression vector pSKOmpFKm. Then, *E. coli* XL1-Blue strain was transformed with the recombinant expression vector pSKOmpFKm, and then the recombinant expression vector pSKOmpFKm containing kanamycin resistant gene was obtained therefrom(<u>see</u>: Fig. 3). Figure 3 represents a genetic map of recombinant expression vector pSKOmpFKm. As shown in Fig. 3, the recombinant expression vector pSKOmpFKm contains *ompF* gene and promoter, which are derived from *E. coli* BL21(DE3) strain, and, kanamycin resistant gene is inserted between *ompF* promoter and 5'-terminal of *ompF* gene, by which *ompF* gene is not expressed. PCR was performed using the recombinant expression vector pSKOmpFKm as a template and primer 3 and primer 4 to obtain DNA fragment comprising ompF gene, its promoter and kanamycin resistant gene inserted between them. The DNA fragment was introduced to *E. coli* BL21(DE3) strain transformed with pTrcEBG and transformants were screened on LB plate containing ampicillin and kanamycin. In order to remove expression vector pTrcEBG from the transformants, the transformants was subcultured in LB media five-times for 2 days, and then spreaded and incubated on LB plate containing kanamycin to screen the transformants not growing on the plate. Genomic DNA of the said selected *E. coli* strain was purified to confirm whether kanamycin resistant gene was inserted between *ompF* promoter and *ompF* gene: i.e., PCR was performed using the purified genomic DNA as a template and primer pair of primer 3 and primer 8: 5'-GATCGGAATTGATTTGAGTTTCC-3'(SEQ ID NO. 8), and amplified DNA fragment was sequenced. Then, PCR was performed using the purified genomic DNA as a template and primer pair of primer 7: 5'-CCACAGCAACGGTGTCGTCTG-3'(SEQ ID NO. 7) and primer 9: 5'-ATCTTTATCTTTGTAGCACTTTCAC-3'(SEQ ID NO. 9), and amplified DNA fragment was sequenced. Sequencing of both DNA fragments revealed that kanamycin resistant gene was located between *ompF* promoter and *ompF*

gene. The said transformed strain was designated as "*E. coli* BL101".

Example 3: Development of expression system of *ompF* gene

In order to express OmpF protein in *E. coli* BL101 recombinant strain prepared in Example 2, three recombinant expression vectors were constructed, respectively.

First, an expression vector of *ompF* gene was constructed employing T7 promoter that is a strong inducible expression promoter. PCR was performed using genomic DNA of *E. coli* BL21(DE3) as a template and primer 4 and primer 10: 5'-GCGAATTCATATGATGAAGCGCAATATTCTG-3'(SEQ ID NO. 10). The amplified PCR product was digested by *Nde*I and *Bam*HI and cloned into an expression vector pET21c(Novagen, U.S.A.) to construct a recombinant expression vector pEDOmpF3. *E. coli* XL1-Blue strain was transformed with pEDOmpF3 and the recombinant expression vector pEDOmpF3 was obtained therefrom(see: Fig. 4). Figure 4 represents a genetic map of recombinant expression vector pEDOmpF3.

Secondly, an expression vector of *ompF* gene was constructed employing Trc promoter that is an inducible expression promoter. PCR was performed using genomic DNA of *E. coli* BL21(DE3) strain as a template and primer 4 and primer 11: 5'-GCGAATTCCATGGTGAAGCGCAATATTCTGGCAG-3'(SEQ ID NO. 11). The amplified PCR product was digested by *Nde*I and *Bam*HI and cloned into an expression vector pTrc99A to construct a recombinant expression vector pTrcOmpF4. *E. coli* XL1-Blue strain was transformed with pTrcOmpF4 and the recombinant expression vector pTrcOmpF4 was obtained therefrom(see: Fig. 5). Figure 5 represents a genetic map of recombinant expression vector pTrcOmpF4.

Thirdly, an expression vector pOmpF6 comprising OmpF promoter constructed in Example 2 was used as an expression vector of *ompF* gene.

The said three recombinant expression vectors(pEDOmpF3, pTrcOmpF4 and pOmpF6) were used to transform *E. coli* BL101 strain prepared in Example 2. Transformed recombinant strains were screened on LB plate containing ampicillin and kanamycin. In order to select the most efficient secretion system that produces OmpF proteins on the cell surface, each transformed recombinant *E. coli* was cultured in 50 mL of R/2 media{$(NH_4)_2HPO_4$ 2g/L, KH_2PO_4 6.75g/L, citric acid 0.85g/L, $MgSO_4 \cdot 7H_2O$ 0.7g/L, 5M HCl/L, $FeSO_4 \cdot 7H_2O$ 10g/L, $ZnSO_4 \cdot 7H_2O$ 2.25g/L, $CuSO_4 \cdot 5H_2O$ 1g/L, $MnSO_4 \cdot 5H_2O$ 0.5g/L, $Na_2B_4O_7 \cdot 10H_2O$ 0.23g/L, $CaCl_2 \cdot 2H_2O$ 2g/L, $(NH_4)_6MO_7O_{24}$ 0.1 g/L, glucose 10 g/L} at a temperature of 37°C.

1M IPTG(isopropyl-β-thiogalactoside, final concentration) was added to induce expression of *ompF* gene when $O.D._{600}$ of culture of the transformant harboring pEDOmpF3 and pTrcOmpF4 reached at 0.7. *E. coli* BL21(DE3) and *E. coli* BL101 were also cultured under the same condition for the control group. Outer membrane protein fractions were prepared from the culture of each strain by the method described in Example 1, and analysed by SDS-PAGE to compare expression levels of OmpF protein. *E. coli* BL101 transformed with pOmpF6 vector produced and accumulated OmpF protein in the outer membrane whose expression level was similar to that of *E. coli* BL21(DE3), a parent strain. From the said results, it was clearly demonstrated that OmpF promoter is the most preferred for the expression of OmpF-fused protein.

The present inventors designated *E. coli* BL101 transformed with a recombinant expression vector pOmpF6 as "*Escherichia coli* BL101/pOmpF6", and deposited with the Korean Collection for Type Cultures(KCTC, #52 Oun-dong, Yusong-ku, Taejon 305-333, Republic of Korea), an international depository authority, as Accession No. KCTC 1026BP on June 1, 2001.

<u>Example 4</u>: Construction of OmpF-β-endorphin expression

vector

β-endorphin protein consists of 31 amino acids, and gene encoding β-endorphin consists of 93 nucleotides(see: Takahashi H. et al., FEBS Lett., 135:97-102, 1981).

To prepare the gene coding for β-endorphin, primer 12: 5'-ACCGCCATACCTTCCCTCGATGAACTGGTAAACGATA-3'(SEQ ID NO. 12), primer 13: 5'-GGAAGGTATGGCGGTTTCATGACCAGCGAAAAAAGCCAGAC-3'(SEQ ID NO. 13), primer 14: 5'-CGCGTTTTTAAACAGGGTCACCAGCGGGGTCTGGCTTTTTTCGC-3'(SEQ ID NO. 14), primer 15: 5'-CCCTGTTTAAAAACGCGATCATCAAAAACGCGTATAAAAAAG-3'(SEQ ID NO. 15), and primer 16: 5'-GCGGATCCCTATTATTCGCCTTTTTTATACGCGTTTTTG-3'(SEQ ID NO. 16) were synthesized, respectively, and used for PCR. PCR was also performed using genomic DNA of *E. coli* BL21(DE3) as a template and primer 10 and primer 12 as primers. Both DNA fragments amplified by the above PCRs were mixed with primer 10 and primer 16, and then PCR was performed again to obtain PCR fragments containing a gene encoding four amino acids which is recognized and cleaved by Factor Xa, and β-endorphin gene fused with *ompF* gene. The said PCR fragments were digested with *Bgl*II and *Xba*I and ligated into an expression vector pOmpF6 to construct a recombinant expression vector pOmpF6βE. *E. coli* XL1-Blue strain was transformed with pOmpF6βE and the recombinant expression vector pOmpF6βE was obtained therefrom(Fig. 6). Figure 6 represents a construction scheme and a genetic map of a recombinant expression vector pOmpF6βE. The nucleotide sequence of β-endorphin gene fused with *ompF* gene in the recombinant expression vector pOmpF6βE is 5'-TATGGCGGTTTCATGACCAGCGAAAAAAGCCAGACCCCGCTGGTGACCCTGTTTAAAAACGCGATCATCAAAAACGCGTATAAAAAAGGCGAATAA-3'(SEQ ID NO. 18), which expresses Tyr Gly Gly Phe Met Ala Ser Glu Lys Ser Gln Ala Pro Leu Val Ala Leu Phe Lys Asn Ala Ile Ile Lys Asn Ala Tyr Lys Lys Gly Glu Stop(SEQ ID NO. 17).

E. coli BL101 strain was transformed with the recombinant expression vector pOmpF6βE and transformants were screened on LB plate containing ampicillin and kanamycin.

Example 5: Extracellular production of OmpF-β-endorphin fusion protein

E. coli transformant prepared in Example 4 was inoculated into 1.8L of R/2 media and cultured at 37°C by fed-batch culture. The substrate was consisted of glucose 700g/L and $MgSO_4 \cdot 7H_2O$ 20g/L, and fed into culture media at a speed of 10mL/min to keep glucose concentration at 0.7g/L if pH of culture media increased above 6.88, and air and oxygen were supplied automatically to keep dissolved oxygen at 40%(v/v). After 17.5 hours of culture, optical density at 600nm of the culture was measured as 150.5 and dry weight of cells was 54.1g/L. To measure the amount of fusion proteins, culture media collected at regular intervals were centrifuged, and the supernatants were analysed by electrophoresis, which showed that OmpF-β-endorphin fusion protein of 40kDa was accumulated in culture media and the accumulated amount of OmpF-β-endorphin fusion protein was increased depending on the culture time. Final amount of OmpF-β-endorphin fusion protein was 4.64 g/L, which corresponded to 45% of total proteins in the culture media.

Example 6: Purification of β-endorphin produced extracellularly

OmpF-β-endorphin fusion protein accumulated in the culture media in Example 5 was purified: First, 50mL of culture media was centrifuged to remove bacterial cells. Then, OmpF-β-endorphin fusion protein was purified from the supernatant by anion-exchange chromatography, where Q2-column(BIO-RAD Co., U.S.A.) was used as anion-exchange

resin and 50mM Tri-HCl(pH 7.0) buffer solution was used as mobile phase and flow rate was 1mL/min. OmpF-β-endorphin fusion proteins were eluted with a linear gradient of 0 to 1 M NaCl. Total amount of OmpF-β-endorphin fusioin proteins eluted at 0.45M of NaCl was 89.1mg. NaCl was removed from OmpF-β-endorphin fusion proteins by dialysis. In order to remove OmpF protein from OmpF-β-endorphin fusion protein, Factor Xa and OmpF-β-endorphin fusion protein were mixed at a ratio of 1:200(w/w) and incubated at 23°C for 12 hours. Then, β-endorphin protein was purified by reverse-phase HPLC, where Microsorb-MV C_{18} column(4.6 x 250 mm, Varian, U.S.A.) was used as HPLC column and 0.1%(v/v) TFA(trifluoroacetic acid) solution was used as mobile phase and flow rate was 1mL/min. Elution of proteins was monitored at 280 nm with a UV detector(<u>see</u>: Table 1).

<u>Table 1</u>: Purification of β-endorphin

Purification Step	Volume (mL)	Total Protein (mg)	Fusion Protein (mg)	β-endorphin (mg)	Yield (%)	Purity (%)
Culture Medium	50	515	232	20.3	100	3.9
Anion-exchange Resin	63	118.8	89.1	7.8	38.4	5.9
RP-HPLC	12	2.8	-	2.8	13.8	>99

As shown in Table 1 above, 2.8mg of β-endorphin was purified by the technique of HPLC. Further, N-terminal sequencing of purified β-endorphin revealed that the amino acid sequence is Tyr-Gly-Gly-Phe-Met-Thr-Ser-Glu-Lys, which corresponds with N-terminal amino acids of β-endorphin.

As clearly illustrated and demonstrated as above, the present invention provides an expression vector comprising genes encoding OmpF and desired protein, *E. coli*

transformed with the expression vector, and a method for extracellular production of desired proteins by employing the same. The recombinant expression vector of the invention comprises ampicillin-resistant gene, OmpF promoter and OmpF gene. In accordance with the invention, a desired protein can be produced extracellularly by simpler method than conventional methods in a manner that: secretory production of OmpF fusion protein begins simultaneously with growth of cells through constitutive expression employing OmpF promoter, and as the concentration of cells increases, the amount of secretory production of the protein also increases continuously. Therefore, desired proteins can be produced in large quantities by a high concentration culture of cells.

While the present invention has been shown and described with reference to particular embodiments, it will be apparent to those skilled in the art that certain changes and modifications can be made to this invention without departing from the spirit or scope of the invention as it is set forth herein.

INDICATIONS RELATING TO DEPOSITED MICROORGANISM

OR OTHER BIOLOGICAL MATERIAL

(PCT Rule 13*bis*)

A. The indications made below relate to the deposited microorganism or other biological material referred to in description On page 12, lines 29-35.	
B. IDENTIFICATION OF DEPOSIT	Further deposits are identified on additional sheet
Name of depositary institution Korean Collection for Type Cultures (KCTC)	
Address of depositary institution (including postal code and country) Korea Research Institute of Bioscience and Biotechnology(KRIBB) #52, Oun-dong, Yusong-ku, Taejon 305-333, Republic of Korea	
Date of deposit June 01, 2001	Accession Number KCTC 1026BP
C. ADDITIONAL INDICATIONS *(leave blank if not applicable)*	This information continues on an additional sheet
D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE *(if the indications are not for all designated States)*	
E. SEPARATE FURNISHING OF INDICATIONS *(leave blank if not applicable)*	
The indications listed below will be submitted to the International Bureau later *(specify the general nature of the indications e.g., "Accession Number of Deposit")*	

For receiving Office use only	For International Bureau use only
☐ This sheet was received with the international application	☐ This sheet was received by the International Bureau on:
Authorized officer	Authorized officer

WHAT IS CLAIMED IS:

1. An expression vector pOmpF6 comprising an ampicillin resistant gene, OmpF promoter and OmpF gene, which is represented as a genetic map of Figure 2.

2. *Escherichia coli* BL101/pOmpF6(KCTC 1026BP) transformed with the expression vector pOmpF6 of claim 1.

3. A method for extracellular production of desired protein employing an expression vector pOmpF6, which comprises the steps of:

(i) introducing a gene encoding oligopeptide which is recognized and cleaved by a proteolytic enzyme and a gene encoding desired protein into the expression vector pOmpF6 to construct a recombinant expression vector producing the desired protein extracellularly;

(ii) transforming a host microorganism lacking OmpF gene with the recombinant expression vector to obtain a transformed microorganism;

(iii) culturing the transformed microorganism to produce OmpF-fused protein from the culture; and,

(iv) treating the fused protein with a proteolytic enzyme and obtaining the desired protein.

4. The method of claim 3, wherein the proteolytic enzyme is Factor Xa, enterokinase, genenase, IgA protease, intein, thrombin, trypsin, pepsin, subtilisin, or plasmin.

5. The method of claim 3, wherein the desired protein is peptide, enzyme or antibody that can be fused with OmpF.

6. The method of claim 3, wherein the desired protein is β-endorphin.

7. The method of claim 3, wherein the recombinant

expression vector is pOmpF6βE.

8. The method of claim 3, wherein the host microorganism is *Escherichia* sp. or *Samonella* sp.

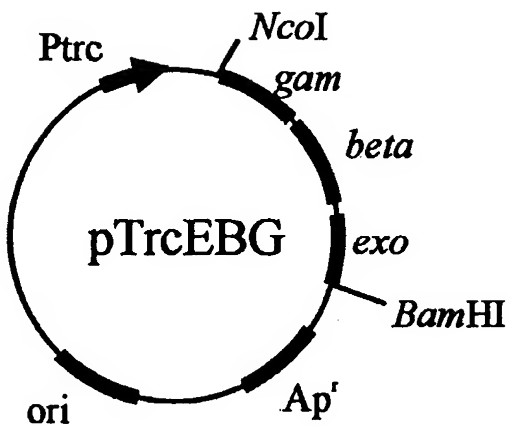

Ptrc
NcoI
gam
beta
pTrcEBG
exo
BamHI
ori
Ap^r

Fig. 1

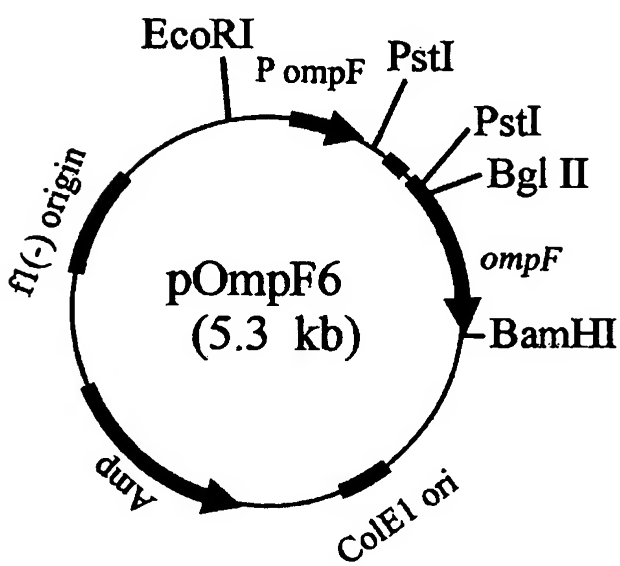

EcoRI
P ompF
PstI
PstI
Bgl II
f1(-) origin
ompF
pOmpF6
(5.3 kb)
BamHI
Amp
ColE1 ori

Fig. 2

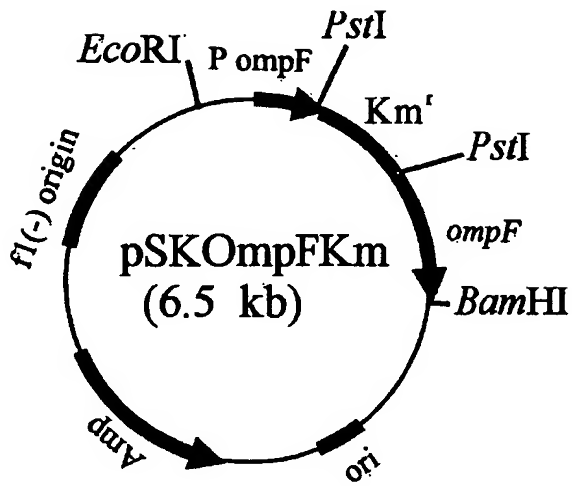

EcoRI
P ompF
PstI
Km^r
PstI
ompF
BamHI
f1(-) origin
pSKOmpFKm
(6.5 kb)
Amp
ori

Fig. 3

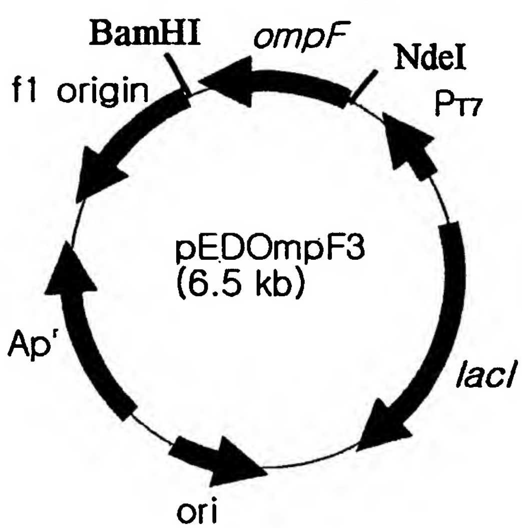

BamHI
ompF
NdeI
f1 origin
P_{T7}
pEDOmpF3
(6.5 kb)
Ap^r
lacI
ori

Fig. 4

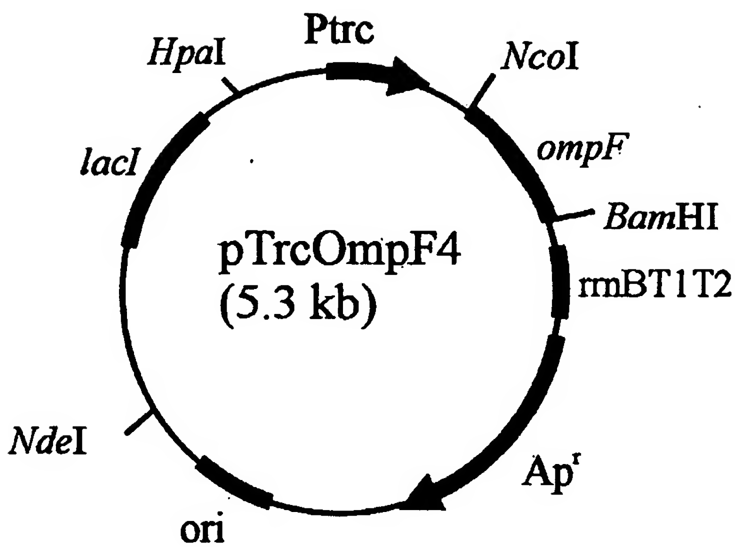

Ptrc
HpaI
NcoI
lacI
ompF
BamHI
pTrcOmpF4
(5.3 kb)
rrnBT1T2
NdeI
Ap^r
ori

Fig. 5

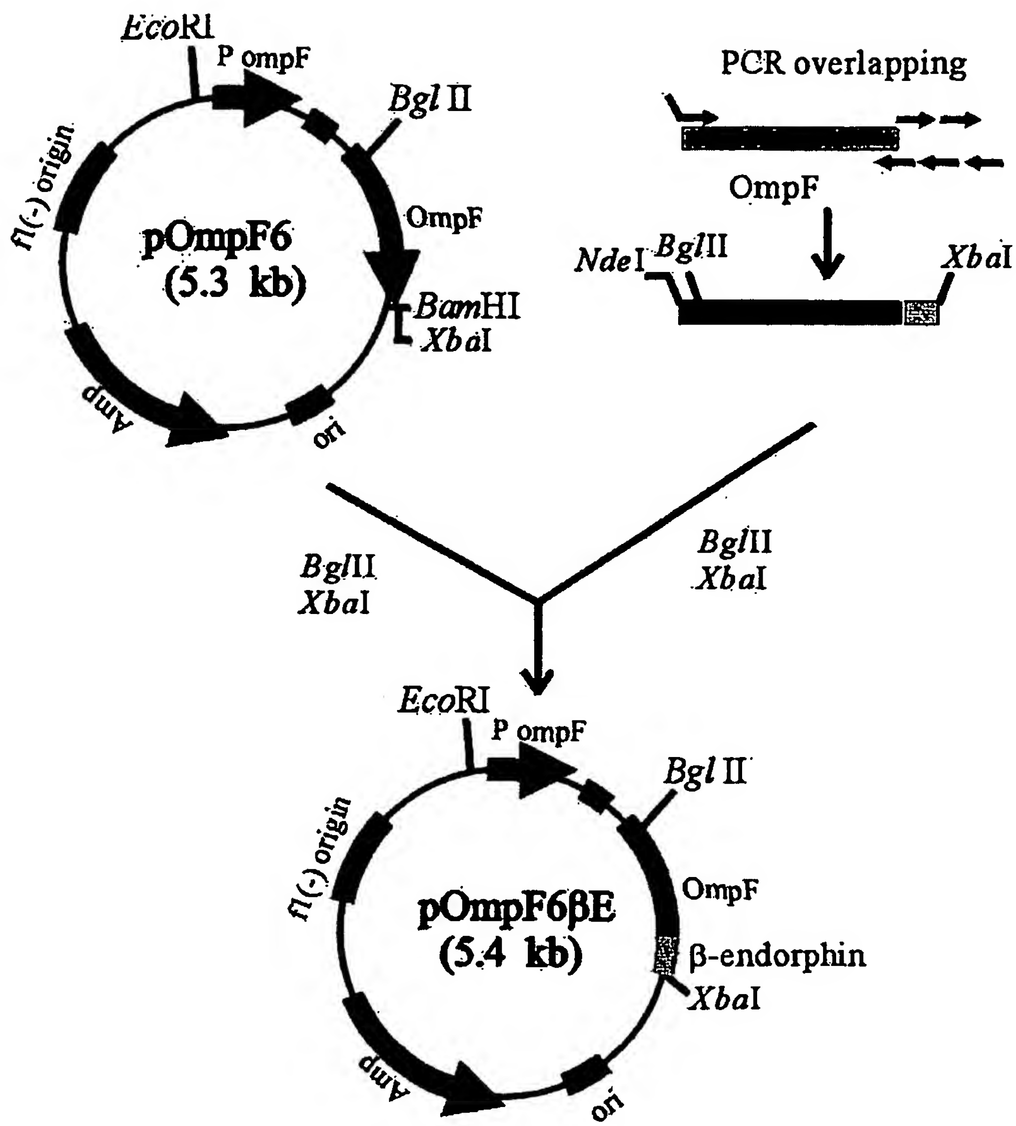

EcoRI
P ompF
Bgl II
fl(-) origin
pOmpF6
(5.3 kb)
OmpF
BamHI
XbaI
Amp
ori
PCR overlapping
OmpF
NdeI
BglII
XbaI
BglII
XbaI
BglII
XbaI
EcoRI
P ompF
Bgl II
fl(-) origin
pOmpF6βE
(5.4 kb)
OmpF
β-endorphin
XbaI
Amp
ori

Fig. 6

SEQUENCE LISTING

<110> Korea Advanced Institute Science and Technology

<120> A Method for Extracellular Producing Target Proteins Employing OmpF in E. coli

<130> DP10655

<160> 18

<170> KopatentIn 1.71

<210> 1
<211> 37
<212> DNA
<213> Artificial Sequence

<220>
<223> primer

<400> 1
cgcgccatgg atattaatac tgaaactgag atcaagc 37

<210> 2
<211> 32
<212> DNA
<213> Artificial Sequence

<220>
<223> primer

<400> 2

cgggatcctc atcgccattg ctccccaaat ac 32

<210> 3
<211> 26
<212> DNA
<213> Artificial Sequence

<220>
<223> primer

<400> 3
cggaattctg gattataccg acgcag 26

<210> 4
<211> 28
<212> DNA
<213> Artificial Sequence

<220>
<223> primer

<400> 4
gcggatcctt agaactggta aacgatac 28

<210> 5
<211> 30
<212> DNA
<213> Artificial Sequence

<220>
<223> primer

<400> 5
cgctgcagtt agaaaaactc atcgagcatc 30

<210> 6
<211> 27
<212> DNA
<213> Artificial Sequence

<220>
<223> primer

<400> 6
gcctgcaggc cacgttgtgt cctcaaa 27

<210> 7
<211> 21
<212> DNA
<213> Artificial Sequence

<220>
<223> primer

<400> 7
ccacagcaac ggtgtcgtct g 21

<210> 8
<211> 23
<212> DNA
<213> Artificial Sequence

<220>

<223> primer

<400> 8
gatcggaatt gatttgagtt tcc 23

<210> 9
<211> 25
<212> DNA
<213> Artificial Sequence

<220>
<223> primer

<400> 9
atctttatct ttgtagcact ttcac 25

<210> 10
<211> 31
<212> DNA
<213> Artificial Sequence

<220>
<223> primer

<400> 10
gcgaattcat atgatgaagc gcaatattct g 31

<210> 11
<211> 34
<212> DNA
<213> Artificial Sequence

<220>

<223> primer

<400> 11

gcgaattcca tggtgaagcg caatattctg gcag 34

<210> 12

<211> 37

<212> DNA

<213> Artificial Sequence

<220>

<223> primer

<400> 12

accgccatac cttccctcga tgaactggta aacgata 37

<210> 13

<211> 41

<212> DNA

<213> Artificial Sequence

<220>

<223> primer

<400> 13

ggaaggtatg gcggtttcat gaccagcgaa aaaagccaga c 41

<210> 14

<211> 44

<212> DNA
<213> Artificial Sequence

<220>
<223> primer

<400> 14
cgcgttttta aacagggtca ccagcggggt ctggcttttt tcgc 44

<210> 15
<211> 42
<212> DNA
<213> Artificial Sequence

<220>
<223> primer

<400> 15
ccctgtttaa aaacgcgatc atcaaaaacg cgtataaaaa ag 42

<210> 16
<211> 39
<212> DNA
<213> Artificial Sequence

<220>
<223> primer

<400> 16
gcggatccct attattcgcc tttttatac gcgtttttg 39

<210> 17
<211> 31
<212> PRT
<213> Artificial Sequence

<220>
<223> Fusion Protein

<400> 17
Tyr Gly Gly Phe Met Thr Ser Glu Lys Ser Gln Thr Pro Leu Val Thr
1 5 10 15

Leu Phe Lys Asn Ala Ile Ile Lys Asn Ala Tyr Lys Lys Gly Glu
20 25 30

<210> 18
<211> 96
<212> DNA
<213> Artificial Sequence

<220>
<223> fusion protein cDNA

<400> 18
tatggcggtt tcatgaccag cgaaaaaagc cagaccccgc tggtgaccct gtttaaaaac 60

gcgatcatca aaaacgcgta taaaaaaggc gaataa 96

INTERNATIONAL SEARCH REPORT

International application No.
PCT/KR02/01547

A. CLASSIFICATION OF SUBJECT MATTER

IPC7 C12N 15/70

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC7 C12N 15/70

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the intertnational search (name of data base and, where practicable, search terms used)
NCBI pubmed database "ompF, vector", Delphion Research Intellectual Property network database "ompF"

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	Appl Microbiol Biotechnol 1992 Jun;37(3):352-7	1-2, 3-8
A	J Biotechnol 1991 Feb;17(2):109-20	1-2, 3-8
A	EP 138644 B1 (Mitsubishi Kasei Co.) Aug. 21, 1991 (21. 08. 1991)	1-2, 3-8
A	JP 59088092 A2 (Mizushima Shoji) May. 21, 1984 (21. 05. 1984)	1-2, 3-8
A	JP 61265092 A2 (Mitsubishi Chem IND LTD) Nov. 22, 1986 (22. 11. 1986)	1-2, 3-8

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevence
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevence; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevence; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents,such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
29 JANUARY 2003 (29.01.2003)	29 JANUARY 2003 (29.01.2003)

Name and mailing address of the ISA/KR	Authorized officer
Korean Intellectual Property Office 920 Dunsan-dong, Seo-gu, Daejeon 302-701, Republic of Korea	LIM, Hea Joon
Facsimile No. 82-42-472-7140	Telephone No. 82-42-481-5590